Gtdap-1 promotes autophagy and is required for planarian remodeling during regeneration and starvation

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Remodeling is an integral component of tissue homeostasis and regeneration. In planarians, these processes occur constantly in a simple tractable model organism as part of the animal's normal life history. Here, we have studied the gene *Gtdap-1*, the planarian ortholog of human death-associated protein-1 or *DAP-1*. DAP-1, together with DAP-kinase, has been identified as a positive mediator of programmed cell death induced by γ-IFN in HeLa cells. Although the function of DAP-kinase is well characterized, the role of DAP-1 has not been studied in detail. Our findings suggest that *Gtdap-1* is involved in autophagy in planarians, and that autophagy plays an essential role in the remodeling of the organism that occurs during regeneration and starvation, providing the necessary energy and building blocks to the neoblasts for cell proliferation and differentiation. The gene functions at the interface between survival and cell death during stress-inducing processes like regeneration and starvation in sexual and asexual races of planarians. Our findings provide insights into the complex interconnections among cell proliferation, homeostasis, and cell death in planarians and perspectives for the understanding of neoblast stem cell dynamics.

autophagy | cell death | planarian | homeostasis neoblast

Remodeling is an integral component of tissue homeostasis and regeneration (1). However, the mechanisms through which this process occurs remain to be fully elucidated. In freshwater planarians (2), remodeling processes occur constantly in a simple tractable model organism as part of the animal's normal life history (3). Planarian tissues contain several differentiated nonproliferating cell types and only one mitotically active cell type called the neoblast, which is believed to be a somatic stem cell. Together with their immediate progeny, neoblasts account for ≈15–25% of all parenchymal cells (4). The proliferative capacity and multipotency of neoblasts allow planarians to have extreme tissue plasticity, which is apparent in their ability to remodel their body pattern in response to extreme damage and starvation (5).

Planarians can regenerate along any body axis in a process involving epimorphosis and morphallaxis. They use morphallaxis to scale their body to the correct new size (reviewed in ref. 5). Moreover, there is a regression of the ovaries, testes, yolk glands, and copulatory apparatus during regeneration of sexual worms that has been described histologically in *Bdellocephala brunnea* (6, 7) and *Dugesia lugubris* (8, 9). In addition, most planarian species can undergo long periods of starvation, during which they are reduced from their normal adult size but maintain perfect scaling. Sexual species also undergo a regression of the gonads on starvation. Gonads and adult size are restored when the animals are fed (10). By analogy to other organisms, apoptosis has been thought to be the mechanism by which cell number is reduced during the remodeling processes associated with the continuous adaptation of the animal to new body sizes during starvation. However, this remains to be demonstrated. Moreover, it remains unclear how energy and resources are made available to dividing cells even during starvation.

One obvious possibility is that differentiated somatic cells provide the raw material through autophagy. Autophagy is a physiological process during which cells turn over organelles and proteins. It has a homeostatic role but is rapidly up-regulated when an organism undergoes architectural remodeling or when cells need to generate intracellular nutrients and energy during starvation (11). It represents a process of cellular self cannibalization and involves an active and controlled rearrangement of subcellular membranes through which cytoplasm and organelles are sequestered for delivery to a lysosome or vacuole and degraded and recycled for synthesis of new macromolecules and ATP (12). The molecular machinery responsible for autophagy is conserved across higher eukaryotes as a survival mechanism under conditions of nutrient deprivation (13, 14). In addition, autophagy has been shown to play a role in tumor suppression (15, 16), pathogen control (17), antigen presentation (18), regulation of organismal life span (19), and developmental processes (14, 19–22). However, the role of autophagy in regulating remodeling of the organism as a whole has yet to be determined, and the involvement of autophagy in programmed cell death (PCD) has been the source of some debate. Although autophagy has long been observed in dying cells during developmental processes (23), it has only recently been shown to be able to induce cell death (24, 25), and the mechanisms involved have only just begun to be elucidated (26).

In this study, we analyzed the role of the ortholog of human death-associated protein-1 (DAP-1), *Gtdap-1*, in planarian remodeling. The DAP-1 protein was originally identified together with the protein DAP kinase (DAP-2 or DAPk) as a positive mediator of PCD induced by γ-IFN in HeLa cells (27), and DAP-2 or DAPk has recently been linked to autophagic cell death (28). Our results indicate that *Gtdap-1* plays a role in autophagy during remodeling processes and offer a perspective on neoblast dynamics. Neoblasts undergoing inappropriate differentiation when a respecification of the body axis is required (for example, after amputation of the head) will either have to be reset by a process likely to involve autophagy to adapt to the new situation or must be removed altogether with differentiated parenchyma cells by PCD. This challenges the traditional view of autophagy as simply a cell-autonomous survival mechanism and highlights the usefulness of planarians as a model system in which to study cellular processes at the organismal level. In particular, planarians are ideal for assessing

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the links between proliferation, cell death, and the mobilization of resources.

Results

Cloning of *Gtdap-1*. An mRNA encoding an ortholog of vertebrate DAP-1 (27) was identified in a PCR-based differential display assay used to isolate differentially expressed genes from regenerating planarians of the species *Girardia tigrina*. The full-length (GenBank accession no. DQ422813) encodes 100 amino acids and contains a *G. tigrina* polyadenylation signal (29) [supporting information (SI) Fig. 5*A*]. Reciprocal BLAST searches of the human genome and the current whole-genome sequence of the closely related planarian *Schmidtea mediterranea* suggest that these genes are the closest genes to each other present in each extant genome.

The putative protein Gtdap-1 was aligned with the human protein DAP-1 and with hypothetical Dap-1 proteins from several organisms predicted by EST or cDNA databases (SI Fig. 5*B*). Gtdap-1, like the human ortholog, is a protein rich in positionally conserved prolines (10%). The conserved sequences in the alignment do not correspond to any known domain. A potential casein kinase II phosphorylation site predicted by both Prosite and ExPASy is the only motif conserved in all organisms; other interesting motifs differ in number and position in different species.

Gtdap-1 Is Up-Regulated During Planarian Regeneration. We investigated the expression pattern of *Gtdap-1* mRNA after amputation in asexual and sexual planarians. Similar data were obtained with planarians cut longitudinally or animals regenerating after fission (data not shown).

In asexual planarians regeneration was induced by double amputation just anterior and posterior to the pharynx (Fig. 1), producing three pieces that regenerated missing parts in ≈12 d at 19°C and reached adult proportions in ≈16 d. Whole-mount *in situ* hybridization (ISH) for *Gtdap-1* was performed at different times of regeneration, using a *Gtdap-1* sense probe as a negative control (Fig. 1*A*, head at 5-d regeneration) and a probe for *Gtops*, a planarian *opsin* homolog expressed in the photoreceptor cells (30), as a positive control (data not shown). *Gtdap-1* was differentially expressed in the parenchyma of the postblastema regions in all of the regenerating pieces but was absent in the blastema itself. At day 1 of regeneration, the gene was highly expressed in a narrow band corresponding to the postblastema and surrounding the wound (Fig. 1 *B–D*). The level of expression increased at the level of the postblastema after 3 d of regeneration as a very intense band surrounding the blastema (Fig. 1 *E–G*). At 5 d of regeneration, we observed high levels of gene expression throughout the parenchyma (Fig. 1 *H–J*). At 7 d of regeneration, there was a clear decrease in expression (Fig. 1 *K–M*). This coincides with the time when the main structures of the new body of the planarian are already formed. At 12 d of regeneration, when the planarians are completely regenerated, medial-regenerating pieces showed only weak expression (Fig. 1*O*). However, expression of *Gtdap-1* remained in the regenerating heads and in the regenerating tails as remodeling and rescaling continues (Fig. 1 *N* and *P*).

ISH in sexual planarians revealed high levels of expression in the gonads, but if the reaction was left to develop longer, we observed the same expression patterns as in asexual worms (SI Fig. 6). After the amputation and 2 d of regeneration, the head pieces expressed the gene in the testes (Fig. 2*A*). This expression became weaker after 5 d of regeneration (Fig. 2*B*) and after 7 d, the gene was no longer expressed in the region of the testes (Fig. 2*C*). These findings are consistent with the histological observation that 8–9 d after beheading, total regression of the testes has occurred in *D. lugubris* (8). We also observed expression of the gene in the ovaries at 5 d of regeneration, and this expression was maintained until 7 d (Fig. 2 *B* and *C*). In tails after 1 d of regeneration, expression was detectable in the testes region proximal to the wound (Fig. 2*D*). Expression in the testes peaked at 3 d after amputation (Fig. 2*E*)
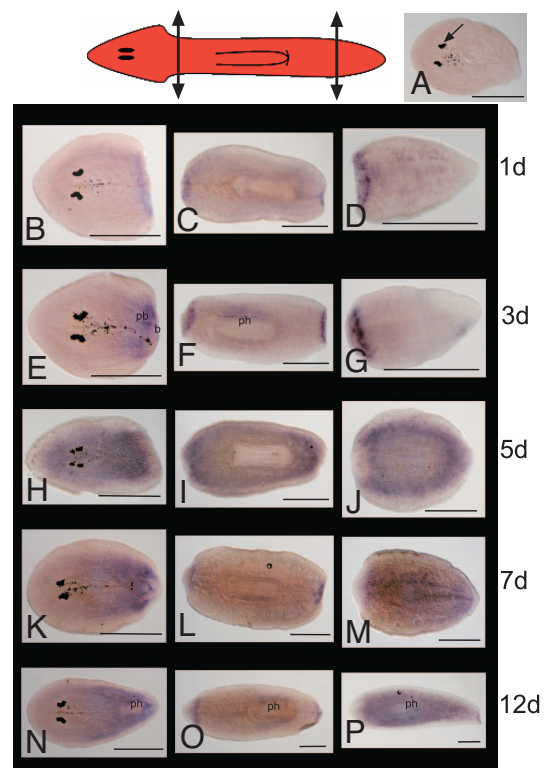
Fig. 1. Whole-mount ISH of *Gtdap-1* in regenerating asexual planarians. Prepharynx and postpharynx amputation sites are indicated in the scheme. (*A*) Negative control using the *Gtdap-1* sense probe in the head of a 5-d regenerating planarian. The arrow indicates the eyes. Head, medial portion, and tail of a planarian at 1 (*B–D*), 3 (*E–G*), 5 (*H–J*), 7 (*K–M*), and 12 d (*N–P*) of regeneration. *Gtdap-1* is expressed in the postblastema (pb) regions at all stages of regeneration but is not expressed in the blastema (b). *N*, *O*, and *P* show regenerated planarians that did not finish remodeling, because the pharynx (ph) is not in its definitive position with respect to the AP axis. *Gtdap-1* expression is maintained in those pieces. (Scale bars, 1 mm.)

and then reduced asymmetrically as regeneration continued (Fig. 2*F*). In double amputations, we consistently saw expression near the anterior amputation decrease first (Fig. 2*G*). Interestingly, amputations close to the ovaries resulted in a very early activation (2 d) in the ovaries (Fig. 2*H*).

Starvation but Not High-Dose γ-Irradiation Up-Regulates *Gtdap-1*. We performed whole-mount ISH for *Gtdap-1* on starving *G. tigrina* asexual and sexual races, with the same controls as described above. After 15 d of starvation, asexual *G. tigrina* showed a clear increase in *Gtdap-1* expression throughout the parenchyma, including the cephalic area (Fig. 2 *K* and *L*), whereas in the sexual race, *Gtdap-1* was highly expressed in the testes, oviduct, yolk glands, and copulatory apparatus (Fig. 2 *I* and *J*), and if the staining reaction was allowed to continue further, it was also seen in the parenchyma (data not shown).

Cell proliferation in planarians can be abrogated by lethal doses of γ-irradiation (31). We irradiated asexual and sexual animals at 10,000 rad and fixed them at 2 h, 7.5 h, 1 d, 2 d, 5 d, or 10 d after treatment and 2 d regenerating organisms and fixed them at 4 d. When whole-mount ISH was performed with *Gtdap-1* on these animals, no expression was observed in any of these cases (SI Fig. 7). At the same time, no significant change was observed in the pattern of expression of the control genes *Gtops* and *Gtwnt-5* (32) (data not shown). With low doses of irradiation (1,000 rad), which do not eliminate all cell proliferation, an activation of *Gtdap-1* is observed (SI Fig. 7). These results suggest that the presence of
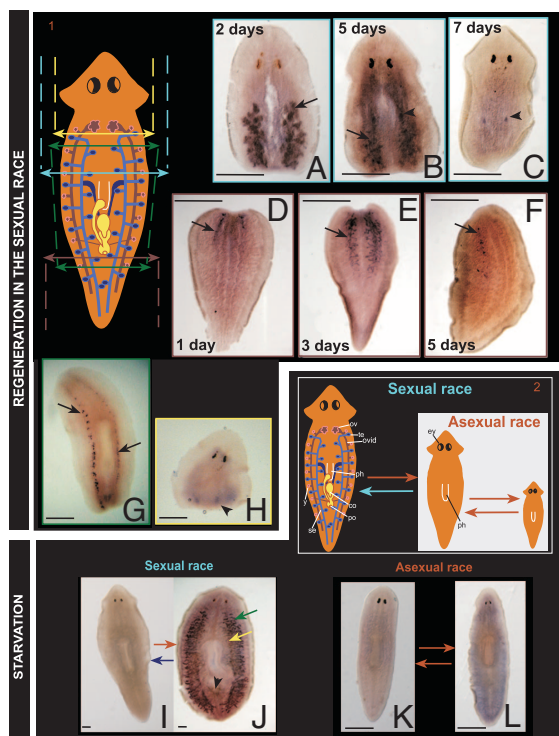
Fig. 2. Expression of *Gtdap-1* mRNA in asexual and sexual planarians during different stress-inducing situations. (Diagram 1) Scheme of a planarian from the sexual race with different amputation planes indicated by arrows. The different colors indicate the corresponding image. (*A–C*) Head after amputation (blue) at 2 d of regeneration shows expression in the testes (arrows) starting from the wound and forming two rows. This expression is weaker after 5 d of regeneration, and at 7 d of regeneration, the gene is no longer expressed in the testes region. Expression of the gene in the ovaries (arrowheads) is shown at 5 d of regeneration, and this expression remains until 7 d. (*D–F*) Tail after amputation (violet) at 1 d of regeneration starts to show high levels of expression in some of the testes closer to the wound; after 3 or 5 d, only the testes (arrows) situated further from the wound express the gene. (*G*) Medial piece (green) of a double amputation after 3 d of regeneration shows a double induction formed from the wounds to the opposite site. Arrows indicate induction of gene expression. (*H*) Amputation close to the ovaries (yellow) results in a very early expression (2-d regeneration) in the ovaries (arrowhead). (Diagram 2) Scheme of a planarian of the sexual race, which resorb the gonads under stress conditions during the process of degrowing and will subsequently continue to shrink in the same way as the asexual race (white box). After removal of the stress condition, the same planarian will grow back, and the sexual race will form new sexual organs. (*I*) No expression in the reproductive system of a standard 7-d starving sexual planarian. (*J*) Expression in the yolk glands and oviduct (green arrow), testes (yellow arrow), and copulatory apparatus (black arrowhead) in a 15-d starved sexual planarian. (*K*) Basal expression in a standard asexual planarian (7-d starvation). (*L*) Increased expression throughout the parenchyma including the cephalic area in 15-d starved asexual planarian. ey, eye; co, copulatory apparatus; ov, ovary; ovid, oviduct; ph, pharynx; po, genital pore; se, seminal duct; te, testes; y, yolk glands. (Scale bars, 1.3 mm.)

proliferating neoblasts and/or regenerative capacity is essential for activation of *Gtdap-1* expression.

Gtdap-1 Is Activated in a Population of Cells Undergoing Autophagy.
Sexual planarians that have been regenerating for 5 d were dissociated and the cells fixed for ISH with probes for *Gtwnt-5* and *Gtdap-1* (SI Fig. 8). *Gtdap-1* was expressed in 44.2% ($n = 234/530$) of all cells, consistent with the high level of expression observed in whole-mount ISH. The population of *Gtdap-1*-positive cells contained many of the planarian cell types (33). Approximately 39% ($n = 134/344$) of all neoblast-like cells (including determined neoblasts and cells in a process of differentiation where the nucleus/cytoplasm ratio is lower than in the neoblast) were positive for
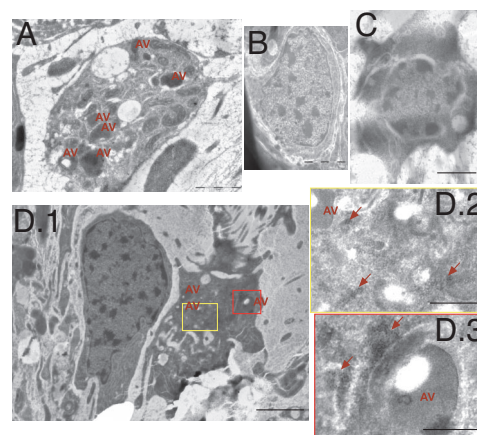
Fig. 3. Micrographs showing the expression of *Gtdap-1* by TEM ISH at the postblastema level of 5-d regenerating sexual planarians. (*A*) Negative control using RNase A showing a differentiated cell undergoing autophagy containing many electron-dense autophagic vesicles (AV) with their characteristic double membranes. (*B*) Neoblast showing no staining for *Gtdap-1*. (*C*) Cell undergoing apoptosis showing some typical features such as cell shrinkage, blebbing of the plasma membrane, maintenance of organelle integrity, and condensation and margination of the chromatin. (*D.1*) The micrograph shows two large cells; the one in the left is a normal differentiated cell, and the one on the right is a cell positive for *Gtdap-1* in a process of autophagy displaying clear cytoplasmic disorganization with electron-dense autophagic vesicles and blebbing of the plasma membrane. (*D.2*) High-magnification image of *D.1* (yellow square) showing gold particles (red arrows) and part of an autophagic vesicle. (*D.3*) High-magnification view of *D.1* (red square) showing the gold particles (red arrows) and an autophagic vesicle that clearly shows the typical double membrane. (Scale bars: 1 μm, *A* and *C*; 2 μm, *B* and *D.1*; and 500 nm, *D.2* and *D.3*.)

Gtdap-1. However, we cannot rule out the possibility that some cells in this group correspond to artifacts resulting from the technical procedure or cells in a late stage of autophagy, because these cells would also be small and have a high nucleus/cytoplasm ratio. In addition, no proliferating neoblasts were observed to express the gene. Approximately 75% ($n = 98/130$) of all differentiated cells were positive. Most of these cells were larger than 30 μm (presumably fixed-parenchyma, gastrodermal, and goblet cells) and contained vacuoles, whereas >96% ($n = 54/56$) of identifiable rhabdite, nerve, epidermal, and muscle cells were negative.

To confirm that *Gtdap-1* is not expressed in proliferating cells, we combined ISH for *Gtdap-1* with immunohistochemistry using an antiphosphorylated histone H3 antibody (anti-H3P) (34) that recognizes mitotic neoblasts (35). This was performed on dissociated cells that were then scanned for coexpression. We conclude there is no colocalization, and that mitotic neoblasts do not express *Gtdap-1* ($n = 0/\approx7,500$) (SI Fig. 8).

Because the whole-mount ISH suggested a role in remodeling and human DAP-1 is known to be involved in cell death processes, we analyzed whether *Gtdap-1*-positive cells displayed features of PCD. ISH for *Gtdap-1* was examined by transmission electron microscopy (TEM) in the postblastema region of planarians at 5 d of regeneration. Negative controls were performed with proteinase K (data not shown) and RNase A (Fig. 3*A* shows an autophagic cell of the negative control). Cells that were positive for *Gtdap-1* by ISH corresponded to differentiated cells containing electron-dense autophagic vesicles with characteristic double membranes, membranous whorls, residual bodies, multivesicular bodies, and engulfed organelles (Fig. 3 *D1–D3*). These results suggest that *Gtdap-1* is expressed in cells undergoing autophagy.

Neoblasts could also be distinguished morphologically (36) and were consistently found not to express *Gtdap-1* (Fig. 3*B*), further suggesting that only determined or differentiating neoblasts express *Gtdap-1*. These cells have to be either eliminated or reprogrammed

to adapt to the new positional value after amputation. Interestingly, cells undergoing apoptosis were negative for *Gtdap-1* (Fig. 3C).

***Gtdap-1* Is Not Involved in Apoptosis.** To assess the role of *Gtdap-1* in PCD, we performed TUNEL assays to detect internucleosomal DNA fragmentation (37) and immunodetection of caspase-3 activation with an antibody against human cleaved caspase-3 that has been shown to cross-react in invertebrates (38). A combination of the two techniques in 5-d regenerating sexual planarians, when the expression of *Gtdap-1* is highest, demonstrated that all TUNEL-positive cells were also positive for cleaved caspase-3 (SI Fig. 9), confirming the role of caspase-3 activation for internucleosomal DNA fragmentation in planarians.

A combination of the TUNEL assay and ISH for *Gtdap-1* revealed no expression of *Gtdap-1* in TUNEL-positive cells from >5,000 dissociated cells of 5-d regenerating planarians in six individual experiments. Moreover, only 2.1% of the total cells were positive for TUNEL. Double labeling of dissociated cells from a 5-d regenerating planarian using ISH for *Gtdap-1* and immunohisto-chemistry with anticleaved caspase-3 antibody showed that only ≈6% of the cells (n = 21/353) were positive for both markers, whereas ≈1.7% (n = 6/353) were positive for cleaved caspase-3 alone (SI Fig. 9). Although it was not possible to perform a triple-labeling experiment, it seems likely that the 2.1% of *Gtdap-1* negative and TUNEL-positive cells correspond to the 1.7% that were labeled with the antibody against cleaved caspase-3 but were *Gtdap-1*-negative.

Although *Gtdap-1* appears not to be expressed in the majority of cells undergoing PCD, as measured by cleaved caspase-3 labeling, but never in the cells undergoing apoptosis, as measured by the TUNEL assay, the possibility remains that there is a temporal correlation between caspase-3-dependent PCD and *Gtdap-1* expression. To assess that possibility, we quantified caspase-3 activity at different days of regeneration (from 1 until 11 d, adults, and 2 weeks starvation) and observed the same dynamics as for *Gtdap-1* expression (Fig. 4C).

RNAi of *Gtdap-1* Affects Remodeling by Reducing Proliferation and Cell Death. To investigate the role of *Gtdap-1* and the associated cell death process in regeneration, we performed a loss-of-function experiment with *Gtdap-1* in the sexual race using RNAi (30, 39, 40). A marked decrease in the speed of regeneration was observed in 12.5% (n = 3/24) of *Gtdap-1* dsRNA-treated planarians, and this decrease was often observed alongside the formation of lesions. These planarians formed a small blastema equivalent to 1 d of regeneration, which was maintained for 5–10 d (Fig. 4 A.1 and A.2), after which time they completed regeneration but developed a deformed head or tail and had motility problems. Of the remaining 21 planarians, 28.6% of regenerating head or tail pieces (n = 12/42) underwent lysis followed by death, a finding that can be explained by the fact that those regenerating pieces have to remodel or undergo more morphallaxis than middle pieces, which are larger and already have almost all of the body structures (Fig. 4 A.3 and A.4). Of all of the fragments that could regenerate, 23.5% (n = 12/51) showed lesions (Fig. 4A.5). We interpret those phenotypes to reflect a failure or reduction in the speed of regeneration alongside remodeling deficiencies in the older parts of the body.

Because of the large number of planarians without a detectable defect (≈50%), we decided to analyze proliferation and cell death rate in animals that survived the *Gtdap-1* dsRNA treatment. Caspase-3 activity in 3-d regenerating *Gtdap1* dsRNA-fed planarians (empty plasmid dsRNA was used as a negative control) was reduced to basal levels similar to those shown in adult standard planarians (Fig. 4C). Proliferation was assessed by labeling with anti-H3P and counting the mitotic cells in the first 750 μm from the wound by confocal microscopy in *Gtdap-1* dsRNA-fed animals (and empty vector as a negative control). A 30% reduction (mean ± SD, 165 ± 15.1 cells compared with 236 ± 44 cells in controls) of total
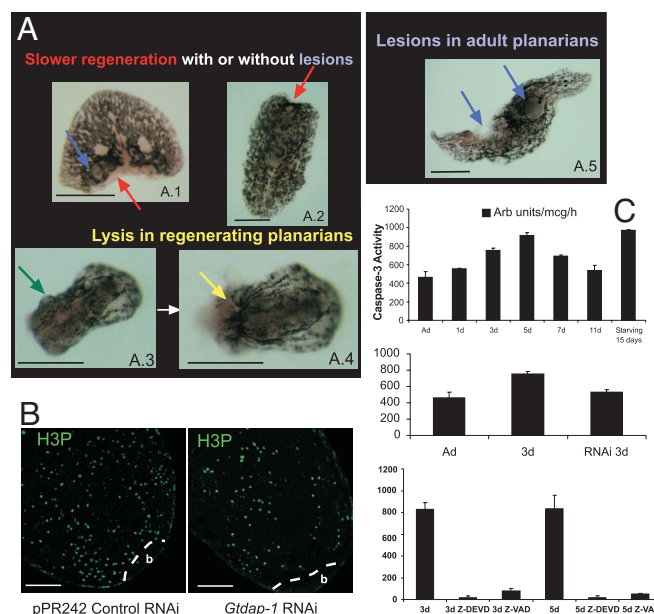
Fig. 4. *Gtdap-1* RNAi experiments. (A) Morphological phenotypes. (A.1) Head after 5 d of regeneration with an abnormally small blastema (red arrow), equivalent to a blastema at 1 d of regeneration, and a lesion (blue arrow). After the day 6, the animal died. (A.2) Tail regenerating for 10 d with an abnormally small blastema (red arrow) equivalent to a 1-d regenerating planarian. After an additional 7 d, it had regenerated a small head. (A.3 and A.4) Head (A.3) showing blisters (green arrow) and the onset of lysis (yellow arrow) after a few hours (A.4). (A.5) Fully regenerated planarian with two lesions (blue arrows). (B) Projections reconstructed from confocal sections of a 3-d regenerating planarian fed with *Gtdap-1* dsRNA or empty vector and labeled with antiphosphorylated histone H3 antibody (H3P). Dashed white lines indicate the border between blastema (b) and postblastema. (C) Caspase-3 activity in protein extracts. Activity is shown as absorbance per microgram of protein per hour (Arb units/μg per hour). (Top) Activity in adults (ad), different days of regeneration, and a planarian starved for 15 d. (Middle) Caspase-3 activity in an adult and planarians fed with dsRNA for the pPR242 empty vector or *Gtdap-1*, both after 3 d of regeneration. (Bottom) Specificity of the assay using two caspase inhibitors, Z-DEVD-FMK and Z-VAD-FMK, in 3- and 5-d regeneration. (Scale bars: 2 mm, A.1, A.3, and A.4; 1 mm, A.2 and A.5; and 250 μm, B.)

mitotic activity was observed in dsRNA-fed 3-d regenerating planarians compared with negative controls (Fig. 4B). The same percentage decrease in mitotic activity was observed in intact planarians (data not shown). The observations show that even when *Gtdap-1* knockdown is not severe enough to give a complete knockdown, it is nonetheless affecting the efficiency of the remodeling process.

Gain-of-Function Mutants in *Gtdap-1* Undergo Massive Cell Death in the Head. A gain-of-function mutant for *Gtdap-1* was generated as described (41) by using the promiscuous transposon *Hermes* and a universal EGFP marker system with three Pax6 dimeric binding sites. The resulting construct, 3xP3-EGFP, is expressed specifically in the photoreceptor cells of all tested phyla (42). A cassette formed by 3xP3-*Gtdap-1* was cloned into the *Hermes*[3xP3-EGFPaf] plasmid to generate the plasmid *Hermes*[3xP3-EGFPaf-3xP3-*Gtdap-1*], which was used for transformation.

Twenty-five adults of the *G. tigrina* sexual race (SI Fig. 10A) and 12 planarians that had been regenerating for 5 d were transformed. The plasmid *Hermes*[3xP3-EGFPaf] was used as a positive control in 20 adults and 6 regenerating planarians, and water was injected and electroporated as a negative control in 10 adults and 5 regenerating planarians. The experiment was performed in duplicate. Transformed planarians were selected by using the EGFP fluorescence marker (n = 12/37 and 14/37) in the eyes (SI Fig. 10B).

Because the phenotype described below prevented maturity and, thus, the possibility of mating and producing sexual progeny, we could obtain only mosaic transgenics. However, progeny obtained through asexual reproduction by architomy (regeneration after spontaneous fission) of transformed planarians were also positive for the EGFP marker.

The *Gtdap-1* gain-of-function phenotype consisted of areas of autolysis or cell death in the cephalic region (100% of the EGFP-positive planarians showed this phenotype). Because the planarians were mosaics, the observed phenotype was quite variable. We could observe lysis in the area of one eye and in different portions of the head (SI Fig. 10 *1b* and *2b*), in both eyes and portions of the head (SI Fig. 10 *5b* and *6b*), in half the head (SI Fig. 10 *3b*), or in the whole head (SI Fig. 10 *4b*). *Gtdap-1* transgenics never grew longer than 0.5 cm and therefore never reached sexual maturity, in contrast to normal adults and controls, which can reach a length of up to 3 cm. The affected area started to regenerate after 4–5 d, with the exception of the planarians with the most severe phenotype, in which lysis of the whole head or half the head occurred (SI Fig. 10 *3c* and *4c*). The blastemas of those animals did not mature further after 2 d and therefore failed to regenerate either the eyes or auricles (SI Fig. 10 *3d* and *4d*). Planarians with a milder phenotype regenerated the affected area, but either one or both regenerated eyes were smaller or one eye was missing (SI Fig. 10 *1c, 1d, 2c, 2d, 5c, 5d, 6c,* and *6d*). All affected planarians slowly remodeled the malformations to become apparently normal transgenic adults, although smaller in size. Interestingly, after 4–7 weeks, cell death occurred again. This led to either death or complete recovery of some planarians, whereas others went through these cycles for the entire 12 months of the experiment.

Discussion

Remodeling is a process that involves a combination of cell proliferation, apoptosis, and autophagy (19). The results of this study show that *Gtdap-1* is involved in remodeling by a process of autophagy during planarian regeneration and starvation. ISH for *Gtdap-1* was examined by TEM showing that the cells expressing *Gtdap-1* morphologically are cells undergoing autophagy. *Gtdap-1* expression was not observed in cells without autophagic morphology (43). The expression pattern of *Gtdap-1* during regeneration coincides both temporally and spatially with the remodeling process. Moreover, 44.2% of cells express the gene at 5 d of regeneration, when the planarian is devoted to remodeling and scaling its body. Finally, the phenotypes of RNAi experiments clearly indicate a role for *Gtdap-1* in remodeling. Even in animals that regenerate correctly without an observable phenotype (presumably due to only partial RNAi knockdown), we measured a 30% decrease in neoblast proliferation and a decrease in caspase-3 activity. Because *Gtdap-1* is never expressed in dividing cells, this effect on proliferation is clearly indirect. We propose that reduction of *Gtdap-1* expression reduces the rate of autophagy, which in turn decreases the amount of resources available to fuel neoblast proliferation and reduces cell death related to autophagy. This results in failing of remodeling or homeostasis, leading to death in the most affected worms.

It is intriguing that 39% of all neoblast-like cells also expressed *Gtdap-1*. We suggest that neoblast-like cells directly contribute to the remodeling process through autophagy. One possibility that remains to be tested is that autophagy may allow them to undergo transdetermination and readjust to their new positional values during regeneration and thus avoid apoptosis that awaits cells that are already phenotypically distinguishable as an inappropriately positioned cell type.

Gtdap-1 expression is also activated during another stress-induced event, starvation. The results of our experiments in starved planarians support the view that autophagy is a response to nutrient deprivation and contributes to survival at both a cellular and organismal level (44). Moreover, we provide further insight into

how sexual planarians eliminate their gonads during stress processes like regeneration or starvation. Early literature from the turn of the century broadly described that "the reproductive organs are resorbed", without providing more details (45, 46), but the choice of words suggested an energetic recycling of the gonads. The results of this study show that during starvation or regeneration the cells of the gonads undergo autophagy, a process that would contribute to a temporary removal of unnecessary structures and at the same time provide energy to the organism to facilitate remodeling and continued homeostasis.

In asexual planarians, it has been described that during starvation, the basal proliferation rate remains constant, and that there is a significant decrease in the number of parenchymal cells (33). Clearly, proliferating and differentiating neoblasts need supplies. Remaining food in the gut and reserves present in the parenchyma cells may be used at first. However, when those resources are used up, nonessential cells for immediate survival like the large differentiated cells in the parenchyma or the cells of the sexual organs would undergo autophagy. It has been shown that fixed parenchyma cells establish intercellular gap junctions with neoblasts (47), and it may be through those connections that unnecessary cells contribute building blocks and energy gained from autophagy to the proliferating and differentiating neoblasts. As a consequence of these processes, the planarian will decrease in size but will be able to maintain its basal proliferation rate.

If new food is encountered, the starved cells will simply produce new organelles. However, if no new food is encountered the cells may reach a point of no return and start to undergo cell death. Although human DAP-1 is involved in cell death events, our results indicate that *Gtdap-1* does not play a role in apoptosis, because examination of apoptotic cells did not reveal expression of *Gtdap-1* either by TEM or in dissociated cells, and we never saw colocalization of *Gtdap-1* expression with TUNEL staining. In contrast to these findings, 6% all cells expressing *Gtdap-1* also expressed the active form of caspase-3. The human active form of caspase-3 is in widespread use as a cell death marker and antibodies against the protein cross-react with invertebrates (38). Our data show that activated caspase-3 colocalizes with TUNEL staining in cells undergoing apoptosis in planarians. We cannot rule out the possibility that the remaining caspase-3-positive cells that are negative for TUNEL are an artifact or a nonspecific effect (48). However, as RNAi for *Gtdap-1* decreases caspase-3 activity and a gain-of-function mutant for *Gtdap-1* displays increased cell death, it may be that the number of apoptotic cells are underestimated by TUNEL staining in our experiments or that some cell death requiring expression of *Gtdap-1* is associated with rapid phagocytosis (49), thereby masking the process of DNA fragmentation. Caspase-3-positive cells expressing *Gtdap-1* may die as a result of autophagic PCD, consistent with previous reports linking caspase-3 to this process (50). This would also be consistent with the morphology of the cells as large differentiated cells in the parenchyma and cells from the sexual organs (44, 51, 52). Furthermore, our results are in accordance with the few studies performed on cell death in planarians, where an increase in vacuolation and autophagic activity was found in the parenchyma of regenerating and starving planarians, resulting in what at that time was referred to as cell autolysis (53–55).

Although the spatiotemporal expression of *Gtdap-1* appears to coincide with neoblast proliferation (56), we do not believe that it is directly involved in this process, because we observed expression of *Gtdap-1* anterior to the eyes, where proliferating neoblasts are not found (Fig. 1*H* and Fig. 2 *J* and *L*) (35, 57) and in large differentiated cells but never in proliferating neoblasts. Moreover, we could not observe colocalization of anti-H3P and *Gtdap-1*. However, there is indeed an indirect relationship between *Gtdap-1* and proliferation, because lethal doses of γ-irradiation prevent expression of the gene but not low doses and RNAi for *Gtdap-1* decreases proliferation. This suggests that autophagy is coupled to

proliferation in planarians during stress-induced events; this correlation could be indirect and simply related to the balance between the "energy supply" by autophagy and "energy demand" created by production of new cells.

In conclusion, our results provide insights into the process of remodeling in planarians, showing that autophagic processes may be essential for the remodeling that occurs during regeneration and starvation. These data highlight the requirement for autophagy in stem cell dynamics.

Experimental Procedures

Animals. *G. tigrina* was used in this work. See *SI Text.*

γ-Irradiation. Planarians were irradiated with a Gammacell 1000 (Atomic Energy of Canada Limited, Mississauga, ON, Canada) for 10,000 and 1,000 rad.

***Gtdap-1* cDNA Cloning.** A differential display assay was used to isolate differentially expressed genes between adult and regenerating *G. tigrina* asexual race (E.S. and C.G.-E., unpublished data). These cDNAs were subcloned into pBluescript + SK (Stratagene, La Jolla, CA). A full-length cDNA clone of 453 bp was obtained by 5′ PCR-RACE (SMART RACE cDNA Amplification kit, Clontech). For details, see *SI Text*.

Cell Dissociation. Cell dissociation was performed by using a modified version of a described protocol (57). For details, see *SI Text*.

ISH Experiments. Digoxigenin-labeled RNA probes were prepared by using an *in vitro* labeling kit (Roche, Basel, Switzerland). Whole-mount ISH and TEM of ISH were performed according to a described protocol (30). ISH on dissociated cells was performed

as for whole mounts, with slight modifications (30, 57). For details, see *SI Text*.

TUNEL Assay on Dissociated Cells. See *SI Text*.

Immunohistochemistry. For whole-mount immunohistochemistry with anti-H3P, planarians were treated with 0.05% colchicine for 7 h and then labeled as described (40). For details on the single and double labelings, see *SI Text*.

Analysis of Caspase-3 Activity. For details, see *SI Text*.

RNAi Experiments. For details, see *SI Text*.

Transformation Assays. Transformation assays were performed according to González-Estévez *et al*. (41). For details, see *SI Text*.

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